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In this final report, we describe our efforts that culminated in the determination of the x-ray crystal structure of a T-cell receptor/MHC molecule (TCR/MHC) complex. Based on the structure, we proposed several common characteristics that all TCR/MHC complexes may have. We prepared and analyzed crystals containing mutant peptides that differ from the wildtype peptide in their immunological properties. We have made progress in determining the structure of another T-cell receptor bound to the same MHC/peptide complex. When finished, these new structures and their comparison to the first TCR/MHC structure should lead to further insights in how T-cell receptors recognize their MHC/peptide ligands.

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Final Report Table of Contents

FINAL REPORT (1 September 1994 - 31 July 1997)

Structural Analysis of the Human T-cell Receptor/ HLA-A2/Peptide Complex

Introduction

Cytotoxic T-lymphocytes play a vital role in the immune response to antigen by their ability to distinguish between self and foreign cells and subsequently to lyse foreign cells and/or to release cytokines. The basis for this differential recognition is the binding of the T-cell antigen receptor (TCR) to cell surface antigens in the form of peptide fragments bound to human leukocyte antigen (HLA) proteins of the major histocompatibility complex (MHC). Some tumors express tumor-specific antigens that can serve to label tumor cells as being "foreign" and that have been shown to direct specific lysis of tumor cells by cytotoxic T-lymphocytes (CTL) in vitro (references 1-5).

At the start of this grant, little was known of the molecular interaction between a T-cell receptor and an HLA protein/peptide complex (MHC molecule) that leads to CTL responses. Over the course of the three years of this postdoctoral fellowship, we studied the interaction between a T cell receptor and the MHC molecule HLA-A2 by x-ray crystallography (6, 7) and gained an understanding of the specific molecular interactions between a particular TCR and the MHC/peptide complex, HLA-A2/tax peptide. This specific recognition of antigen by a T cell through its TCR forms the basis for T cell responses.

Year 1: September 1, 1994-August 31, 1995

In the first year of this grant, we began an x-ray structure determination of a TCR/HLA-A2/peptide complex that would likely yield an understanding of the recognition that is required for specific T-cell responses, and that would have

relevance to the development of cancer immunotherapies using tumor-specific CTLs to control tumor growth.

To obtain sufficient amounts for crystallization, the human TCR and HLA-A2 proteins were produced in bacteria. Abundant HLA-A2 complexed with single peptides was available from previous work (8). Refolding experiments with the TCR obtained from bacterial expression were successful and provided ample TCR protein for crystallization. We prepared x-ray quality crystals of the TCR/HLA-A2/peptide complex and collected a dataset to a resolution limit of 3.3 Å. We began the process of solving the TCR/HLA-A2 structure by molecular replacement techniques. We expected that by using the HLA-A2 structure (9) and the published structures of domains of a murine TCR (10,11) the structure could be solved.

Results

The first-year task for this project was to obtain sufficient amounts of the TCR, since relatively large amounts of protein are needed for crystallization experiments. Our approach was to overexpress the alpha and beta subunits of the TCR in bacteria, to isolate the overexpressed, but insoluble proteins, then to refold the subunits into their native state. The human TCR that we used is directed against the peptide (LLFGYPVYV) derived from the tax protein of human T-lymphotropic virus-type 1 (HTLV-1) and is restricted to HLA-A2 as described more fully in the grant proposal and in ref. 12.

The procedure is described below is the one that was used to produce functional TCR in sufficient yield for crystallography experiments. The experimental data showing the TCR to be functional has been published in ref. 7 (see reprint forwarded at time of publication) and is summarized in brief below.

Refolding by dialysis.

The denatured alpha and beta subunits were separately diluted into a buffer containing 100 mM Tris-HCl, 400 mM L-arginine-HCl, 5 M urea, 2 mM EDTA, pH 8.3 to a protein concentration of 2 uM. Urea was removed by dialysis against the same buffer but lacking the urea and then the buffer was changed by dialysis against 20 mM Tris-HCl, 150 mM NaCl, 0.5 mM dithiothreitol (DTT), pH 7.5. The dilute proteins were concentrated by ultrafiltration with a stirred cell (Amicon Corp.). Since the TCR as found in human cells has a disulfide bond between the alpha and beta subunits, it was possible that this bond would be required for the TCR to fold properly, to be active, and/or to initiate a cellular signal. Therefore, we initially produced a refolded TCR with this inter-subunit disulfide bond. The beta subunit were treated with Ellman's reagent (Sigma Co.) to derivatize the free cysteine at the C-terminus of the protein. Then both alpha and beta were again dialyzed against 20 mM Tris/150 mM NaCl, pH 7.5, to remove the Ellman's reagent and the 0.5 mM DTT. After re-concentration, alpha and beta were mixed at a 1:1 molar ratio to form a disulfide bond by the attack of the free C-terminal cysteine of the alpha subunit on the derivatized, activated C-terminal cysteine of the beta subunit. The alpha/beta TCR protein was again dialyzed, then concentrated and using directly for binding studies.

Binding Assay Using Native Polyacrylamide Gels

The refolded TCR was tested for its ability to bind its specific HLA-A2/tax peptide complex (7). Upon mixing the TCR and HLA-A2/tax proteins and electrophoresing the mixture on native polyacrylamide gels, a new band appeared which differed in mobility from the bands observed when either the TCR or the HLA-A2 protein was electrophoresed alone. Native gels were 15% acrylamide and used the standard Tris/glycine buffers, but without the detergent sodium dodecylsulfate (SDS). The new band appeared to be the complex of TCR and HLA-

A2. No band appeared when the peptide bound to HLA-A2 was an altered tax peptide having position 4 (G -> K) and position 8 (Y -> K) mutations. The complex band was isolated from the gel and re-electrophoresed on a denaturing gel containing SDS. The protein bands for the two HLA-A2 subunits appeared as did a 55 kD band when the gel sample buffer did not contain beta-mercaptoethanol (non-reducing conditions), or as an intense 28 kD band when the gel sample buffer contained beta-mercaptoethanol (reducing conditions). This was evidence that the TCR and HLA-A2 were forming a complex since all the subunits of such a complex were present in the new band on a non-denaturing gel. The denatured alpha and beta subunits have the same mobility on SDS gels which explains the single intense band seen using reducing sample buffer (7). The marked change in mobility upon reduction of the disulfide bonds indicated that the interchain disulfide was being formed.

The dialysis/Ellman's procedure for preparing disulfide-bonded TCR protein was successful in that it yielded protein that specifically bound HLA-A2 complexed with the tax peptide, but the procedure did not yield enough protein at a sufficiently high level of purity for crystallization trials. The protein appeared heterogeneous by non-reducing SDS gel electrophoresis. There may be several reasons for this heterogeneity. We have since learned that the Calpha domain is an unusual immunoglobulin domain that may be less stable (13). Our treatment of alpha with DTT may have opened the disulfide bond in Calpha and allowed either of those two cysteines to react with the activated beta subunit in the Ellman's reagent protocol. The Cbeta domain has a free cysteine at the surface of the protein (10) which may also react in the Ellman's coupling reaction.

A strategy to prepare TCR protein that does have a disulfide bond between the subunits may be a modified one to that described above. If the alpha subunit (instead of beta) is treated with Ellman's reagent to derivatize the free cysteine at

the C-terminal, the oxidizing environment of the Ellman's treatment may stabilize the unstable disulfide bond in Calpha. Then, if the mutant beta subunit (see below) lacking the free cysteine in Cbeta is used, the specific linkage between the C-terminal cysteines of both subunits might be achieved. Performing the experiment in this manner may yield fewer side reactions which can lead to a greater heterogeneity of the preparation.

The Interchain Disulfide is Not Necessary for Binding.

The cysteines involved in the interchain disulfide bond may lower yields of properly folded protein, since during refolding unpaired cysteines can catalyze disulfide exchange to form non-native disulphide bonds or can become oxidatively damaged, especially in the presence of protein denaturant. To test whether TCR interchain disulfide bond was required for complex formation, iodoacetamide (IAA) was added to the refolded alpha and beta subunits. IAA covalently modifies free cysteines, making them no longer reactive in disulfide bond formation. After mixing the IAA-treated TCR subunits, no disulfide bond was evident by SDS gel electrophoresis (reduced and nonreduced). A new complex band still appeared on native gels after mixing the IAA-treated TCR subunits and the HLA-A2/tax protein. This implied that the disulfide bond between alpha and beta is not necessary for binding to HLA-A2. To remove the unneeded cysteines, we moved the stop codons in the plasmid constructions for both the alpha and beta proteins. For alpha, the single C-terminal cysteine was replaced by a stop codon by polymerase chain reaction-mediated mutation. For beta, the single C-terminal cysteine was replaced by a stop codon in an analogous manner. In addition for beta, the free cysteine within the constant domain was replaced by an alanine residue. These stop codon changes also altered the lengths of both alpha and beta, so that they could now be distinguished by their differing mobilities on SDS denaturing gels (7).

Refolding by Dilution

Our current optimized procedure for refolding the anti-tax TCR by dilution is as follows. Alpha and beta inclusion body protein is prepared in 50 mM 2-(N-morpholino)ethanesulfonic acid (MES), 8 M urea, 2 mM NaEDTA, 0.1 mM DTT at approximately 10-20 mg/ml as determined by a Commassie dye-binding assay (Biorad). Equal amounts of alpha and of beta (~30 mg) are diluted into 6 M guanidine-HCl, 10 mM NaAcetate, 2 mM EDTA pH 4.5 and kept at room temperature. A liter of refolding buffer is prepared consisting of 100 mM Tris-HCl, 400 mM L-arginine-HCl, 2 mM NaEDTA, 6.3 mM cysteamine, 3.7 mM cystamine and adjusted to pH 8.5 at room temperature. The refolding buffer is brought to 10°C. The alpha/beta guanidine solution is injected into the refolding buffer through a #27 needle while stirring the refolding buffer vigorously. The refolding TCR solution is kept at 10°C for 24 h. It is then dialyzed against 10 L of 100 mM urea at 4°C for 24 h and then against a new 10 Lof 10 mM Tris-HCl, 100 mM urea, pH 7.5 for 24 h more. The dialyzed protein solution is loaded on to a column of DE52 anion exchange resin (Whatman) in 10 mM Tris-HCl, pH 7.5 and the protein is eluted into ~50 ml with 10 mM Tris-HCl, 300 mM NaCl, pH7.5. After concentration in a Centriprep-30 (Amicon), the TCR is purified on Sephacryl-200 and Superdex-75 gel filtration columns (Pharmacia). The purified protein is concentrated to 50-75 mg/ml and buffer-exchanged to 10 mM Tris-HCl, pH 7.5.

Crystallization and Data Collection

The TCR (75 mg/ml) and HLA-A2/tax (12 mg/ml) were mixed at a 1:1 molar ratio and set up in a hanging drop vapor diffusion crystallization experiment. A screen of crystallization conditions was used (ref. 14). Crystals appeared in 10% polyethylene glycol (PEG) 6000, 100 mM MgAcetate, 50 mM NaCacodylate, pH 6.5. These crystals were reseeded (ref. 15) and large (200 x 200 x 100 um) crystals were obtained. The larger crystals were mounted in an glass capillary tube for diffraction.

Diffraction was observed to about 3.5 Å resolution. Crystals were placed into the above buffer plus 20% glycerol and frozen at -160°C . Diffraction was observed to about 3.3 Å and a complete dataset was measured to 3.5 Å (60% complete 3.3-3.4 Å). The spacegroup is C2, with cell dimensions, a=228.2, b=49.6, c=94.7 Å, and beta=91.3°.

Conclusions at the End of Year 1

In the first year of the project we have refolded a TCR and showed that it binds to its specific peptide/HLA-A2 complex. We have an ample supply of protein for crystallographic studies. At present we are solving the structure of the TCR/HLA-A2 complex to 3.3 Å resolution. This is moderate resolution and we will attempt to obtain higher resolution data. With the laboratory rotating anode x-ray source, the limit of diffraction of these crystals appears to be ~3.3 Å, though we are continuing to search for a modified set of conditions for freezing the crystals (buffers, temperature, protocol, etc.) that will lead to higher resolution data. We have applied for time at the Cornell High Energy Synchrotron Source (CHESS) to use the high intensity x-rays generated there to obtain better data from our present crystals. We continue to experiment in finding new crystal forms that will allow higher resolution data to be collected in our laboratory or elsewhere.

It is possible that the phases yielded by the molecular replacement solution of the HLA-A2/peptide structure will not yield an interpretable electron density map, especially at the current resolution of our data. Several strategies to overcome this are planned. (a) Heavy atom derivative data will be obtained to provide phases through standard crystallographic methods. The unique free cysteine in the constant region of beta can be engineered back into the protein for reacting with a mercury compound. (b) Seleno-methionine will be incorporated into one or more or the four subunits making up the TCR/HLA-A2 complex to provide a heavy

atom derivative and anomalous scattering for phase improvement. The large seleno-methione residues also act as "landmarks" and aid in the tracing of the polypeptide chain through experimental electron density. (c) Obtaining data from a second crystal form would allow cross-crystal averaging to improve the electron density map to a more interpretable quality.

Year 2: September 1, 1995-August 31, 1996

In the second year of this grant, we determined the x-ray structure of a TCR/HLA-A2/Tax peptide complex. This has furthered our understanding of the recognition that is required for specific T-cell activation and may better inform the development of tumor immunotherapies using tumor-specific CTLs to control tumor growth.

In October 1995, Partho Ghosh, who was a postdoctoral fellow in the laboratory, joined the project. He was experienced in X-ray crystallographic techniques and analysis. In this final report, Partho Ghosh and I (David N. Garboczi) collaborated in performing the work described in Year 2 and in Year 3.

In the first year of this grant we had obtained crystals of the TCR/MHC complex. The human TCR and HLA-A2 proteins were produced in bacteria; procedures for producing abundant HLA-A2 complexed with single peptides had been available from my previous work (8). Refolding experiments with the TCR obtained from bacterial expression had been successful and ample TCR protein was in hand (7).

Results

We had some difficulty in freezing diffraction-quality crystals reproducibly, but found improved conditions for freezing the crystals using 20% glycerol and 50 mM TrisCl, pH 8.5 with 10% PEG 6000 and 100 mM Mg acetate. Crystals frozen

under these conditions reproducibly diffracted X-rays. Microseeded crystal growth was performed at a PEG concentration of 7.5%, which appeared to increase the size and number of crystals obtained. The size of a typical crystal used for data collection was 100 x 200 x 700 μm.

We confirmed the C2 spacegroup based on the dataset described above. Analysis by XDS (16) autoindexing and inspection of integrated, but unreduced intensities in HKLVIEW (17) showed the spacegroup to be the centered monoclinic group, C2. The R_{sym}= 12.4 % and the mosaicity was estimated to be 0.6 deg full-width at half-height from analysis of a rocking curve. From the cell constants, one complex was estimated to be in the asymmetric unit with a 56% solvent content.

Using the program AMORE (17), a molecular replacement (MR) solution with the HLA-A2/Tax model (9) was readily found with a correlation coefficient (cc) of 0.29 in the rotation search (next highest cc = 0.16) and with a cc in the translation search of 0.29, and an R-factor (Rf) of 51% (next highest cc = 0.21, Rf = 54%). Translation solutions were found with a mouse β subunit model (ref. 10) with a cc of 0.37 and a Rf of 0.49) and with a mouse Vα domain model (ref. 11) with a cc of 0.44 and a Rf of 46.6% (next highest cc= 0.42, Rf- 47.4%). Rotation searches were done with 10-3.8 Å data and translation searches were done with data from 15-3.8 Å. The β and α search models were poly-alanine except where residues were conserved. The mouse β subunit has 69 % sequence identity and Vα has 34 % sequence identity with this human TCR (data not shown). Some loops in Cβ and in Vα were deleted for the search. Molecular replacement translation functions with α and β domains were done with HLA-A2 fixed. The MR placement of the V regions "looked right" as they independently were positioned above the binding site and formed an eight-stranded beta barrel at their interface, being very similar to the structure of the V-V portion of Fab antibody fragments.

A 2Fo-Fc electron density map using just HLA-A2 as model to generate Fcalcs and solvent-flattened and histogram-matched with the program DM (17) revealed density above the peptide binding site, but this and other 2Fo-Fc maps using various combinations of the A2 and TCR domains as models were poorly interpretable for portions of the protein that were not part of the models.

Synchrotron data collection I

We planned to obtain a better X-ray dataset and believed that we would likely solve the structure with higher resolution data and the HLA-A2, murine TCRα, and TCRβ models. We also planned to collect data from crystals soaked in mercury and platinum to make heavy atom derivatives for use in multiple isomorphous replacement (MIR). A mutation was made in the Tax peptide (sequence: LLFGYPVYV) by changing the leucine at position 1 to a cysteine (P1cys); mercury reacts covalently with cysteines. The TCR/HLA-A2 gel shift still occurred with the P1cys peptide. The HLA-A2/P1cys protein was ready at the last minute before going to the synchrotron and had not been purified. At the CHESS synchrotron, we were able to purify the protein by precipitating impurities with 20 mM Mg acetate at the pH 6.5 of the crystallization solutions. Micro-seeded drops with TCR and the precipitation-purified HLA-A2 bearing the mutant peptide yielded crystals there, at CHESS. They were treated with ethyl mercury phosphate and the mercury derivative data was immediately collected. Native crystals were treated with K_2PtCl_4 to obtain a platinum derivative dataset.

Native data (data resolution limit 2.8 Å) and derivative data (mercury 3.4 Å and platinum 3.6 Å) were obtained at CHESS on an Area Detector Systems Corp (ADSC) charge-coupled device (CCD) X-ray detector, and were integrated and scaled with DENZO/SCALEPACK (Z. Otwinowski, personal communication). The P1cys mercury data was the least mosaic (0.6 %) but not complete (75 %). Native data was 79% complete and the Pt data was 94% complete, but both were highly mosaic

(1.5°and 1.4°, respectively). The mercury position was found by difference Patterson methods. Its location was confirmed in a difference Fourier map phased on HLA-A2; the mercury peak was located on the side chain of P1 of the peptide as we had expected/hoped. The platinum positions were located in difference Fourier maps phased from the protein models. Refinement of heavy atom positions in MLPHARE (17) yielded one major and one minor site (phasing power = 0.94, >1 to 4.9 Å, R_{cullis}= 0.76 to 3.5 Å) for the mercury and one major and four minor sites (phasing power= 0.92, >1 to 4.9 Å, R_{cullis}= 0.82 to 3.5 Å) for the platinum, with an overall figure-of-merit of 0.35 (see Table 1 of the Appendix). The positions and occupancies of the heavy atom positions were re-calculated in MLPHARE using DM-modified phases as reference phases. Solvent-flattened and histogram-matched MIR electron density maps showed broken and ambiguous density above the peptide-binding site. The synchrotron native dataset did not scale well with the first dataset that was collected in the laboratory on a multiwire detector (Siemens) detector (R_{deriv} = 40 %). Rfactor of the derivative here refers to the differences between the two native datasets under discussion.

Synchrotron data collection II

We decided to obtain datasets on additional derivatives to improve the MIR results and also to attempt to obtain a better native dataset. Single and double cysteine mutants of β_2-microglobulin (β_2m) were made by Qing Fan, a graduate student in the laboratory, at positions 52, 67, 91 in β_2m. The mutations were chosen to be accessible to solvent and to be free of crystal contacts by studying the packing model for the TCR/MHC crystal generated from the MR placement of the models. The refolding of HLA-A2 was not impaired by the cysteine mutations in β_2m. To provide an additional mercury site in the hope of generating an additional derivative, an eleven amino acid peptide with cysteine at P1 and P11 and glycine at P10 was made; the TCR/HLA-A2 native gel shift still occurred. Another peptide

was made with a wild-type P1 and mutant P10 and P11. This also yielded a gel-shift with TCR and MHC.

Data was obtained at CHESS with the same CCD detector. Derivative datasets obtained during the second trip to CHESS were named by the position of free cysteines placed for mercury binding: P1cys, Y67C K91C, P1P11, native, K91C (see Table 1). Some of the datasets would not scale well with the other datasets and we found that the data fell into two groups. Within a group the data would scale between datasets and achieve a R_{deriv} of 20-25 %. Between the two kinds of crystals, the data would not scale well (R_{deriv} of 40 %, 35-40% even in the low resolution bins), even though the cell constants are almost the same. The cell constants for the two best native sets are (a=228.4 Å, b=49.4 Å, c=95.7 Å, β= 89.7°; α=229.3 Å, β=49.5 Å, c=96.0 Å, β= 89.6°). For group 2, the Y67C K91C set (called B5) was chosen as "native" (2 mercury atoms) and is phased by the sets: native, K91C, and P1cys. The B5 data is to 2.6 Å with a 1.2° mosaicity and a completeness of 90%. We found that there were rotation and translation differences in domain positions between the two native sets approximately as given:

Domain	Rotation	Translation
α1α2	2.0°	2.9 Å
α3	3.2	4.8
β_2m	0.8	1.5
Vβ	3.2	4.6
Cβ	2.4	3.8
Vα	1.8	2.6

Averaging the two MIR maps generated from the two groups of data was begun. Initial transformations between the seven domains were obtained from the MR solutions. To improve the transformations, domain by domain density

correlation between the two maps was performed with the IMP program from the RAVE package (18). The 2Fo-Fc map using HLA-A2 as model was used in the averaging as a real space contribution to the averaging and in parallel, in phase combination with the MIR phases of each cell to contribute to the starting maps. The HLA-A2 molecular replacement map was allowed to contribute to just its own masks or to all the masks. Averaging was performed in two ways, either phase-combining the averaged phases with the MIR phases at each cycle or not. Current rotation/translation operators were obtained by refining the model into each dataset and determining the tranformation in the graphics program, O (ref. 19).

Two-fold averaged MIR/MR maps were much improved and allowed the positioning and building of all the main chain except the CDR3 regions and most of the side chains of the Vα and Vβ domains. 2Fo-Fc maps using this improved model allowed a convincing trace of the CDR3 regions.

The new space group $P2_1$ with the 11mer peptide had the same cell constants but no centering and therefore had two complexes per asymmetric unit. Deciding between the P2 and $P2_1$ spacegroups was difficult ; there were very few observations of 0k0 reflections since k is along the 49 Å axis. Molecular replacement with AMORE placed HLA-A2 in one of the two positions, but did not find the second position. A difference Fourier using HLA-A2 as model (in only one of the two positions in the asymmetric unit) located the two mercury atoms in the asymmetric unit. The second HLA-A2 model was translated to match the second mercury site. The statistics of the top rotation/translation solution assuming the spacegroup $P2_1$: cc= 0.29, Rf= 51% with the packing model looking possible. The statistics for the top rotation/translation solution assuming the spacegroup P2: cc= 0.25, Rf=52% but the packing model looked physically impossible as the proteins overlapped each other. The $P2_1$ information was included in the real-space averaging protocol thus making the averaging four-fold.

Description of the Structure

The 2.6Å structure (see ref. 6 for more details and figures) of the TCR/Tax/HLA-A2 complex shows that the TCR is oriented diagonally over the HLA-A2/peptide complex, with CDR1 and CDR3 from both Vα and Vβ contacting the peptide. This orientation appears similar to that in the preliminary description of a mouse TCR (2C)/peptide/H-2K^b complex (13). The overall structure of the TCR in the TCR/Tax/HLA-A2 complex is similar to the structure of a Fab. All three variable loops of Vα and CDR3 of Vβ contact conserved and polymorphic positions on the α-helices of the MHC molecule. The interface between TCR and MHC/peptide is in unambiguous electron density, as are the α1, α2, and β_2m domains of the MHC and the Vα and Vβ domains of the TCR.

Figure 1 (see Appendix, ref. 6) shows the TCR/Tax/HLA-A2 complex and how the TCR (top) and MHC molecule (bottom) would span the space between a CTL and virally infected target cell.

When the peptide-binding site of HLA-A2 is viewed from above, the TCR is oriented diagonally across the site (Figure 2 in Appendix, see ref. 6). The TCR third variable loops of both Vα and Vβ meet at the center of the binding site over the center peptide residue Y5. The CDR1 loops are also positioned over the peptide. CDR1α extends from the peptide N terminus to Y5, and CDR1β is over the C-terminal end of the peptide near Y8. The second variable loops are over the MHC molecule, CDR2α over the α2 domain α-helix and CDR2β over the α1 domain α-helix.

Almost the entire Tax peptide is buried in the TCR/MHC interface. The TCR makes contacts with peptide residues L1, L2 and from G4 to Y8. Y5 is bound in a deep pocket at the center of the TCR where the CDR3 loops converge, reminiscent of the structures of peptides complexed with anti-peptide Fabs (reviewed in ref. 20).

The peptide is bound much more deeply in the MHC molecule than it is in the TCR.

The diagonal orientation of the TCR allows the flat surface of the TCR to interact with the peptide by fitting down between the two highest points on the MHC molecule, toward the N terminal of the α1 domain α-helix and toward the N-terminal end of the α2 domain α-helix, a feature of the diagonal orientation also recognized by Nathenson and colleagues (21). The high points of the α2 domain α-helix and the α1 domain α-helix are a topographical feature common to all class I and class II molecules.

No gross conformational change of the TCR was observed in the complex relative to the structures of isolated TCR domains or Fabs that might indicate that it could send information to the cytoplasm that it had bound antigen.

Conclusions

The suggestion that a diagonal orientation allows a general 'interlocking' binding mode between TCR and MHC was first proposed on the basis of mutations on mouse class I molecules (21). In addition to the structural arguments presented here, functional arguments have been made in favor of a general binding mode (refs 22, 23 for example). For example, it would provide a molecular mechanism for an inherent bias of TCR for self-MHC (24) and, coupled with the apparent physical limit on TCR specificity for peptide seen in this structure, helps to explain the ability of one peptide positively to select a nearly normal TCR repertoire (23,25). A general TCR binding mode would simplify the processes of selection during repertoire development and provide an explanation for the magnitude of the alloreactive response. One way that the danger inherent in hypervariable molecules may be controlled is by restricting their opportunity for cross-reactivity to

a single binding mode on MHC molecules, which could be part of the basis of mechanisms that maintain immunological tolerance.

Plans for the Third Year of the Fellowship

As we continue to analyze the TCR/Tax/HLA-A2 structure for additional insights, we are also determining the structures of two related complexes to more fully understand the recognition of the antigenic peptide/MHC complex. We have identified two mutant Tax peptides that alter the TCR recognition of the HLA-A2 complex (see ref. 7 for complete details). One of them, Y5A, allows near wild-type cell-killing by the T cell bearing the anti-Tax receptor, but promotes less than 10% of the wild-type cytokine release by the T cells. Y5A promotes complex formation as seen in a gel shift assay; a shifted band is observed. The partially-activating characteristic of this peptide has led peptides of this sort to be termed "partial agonist" peptides (26). We have TCR/Y5A/HLA-A2 crystals that diffract x-rays. The other peptide contains the mutation Y8A and, in T cell assays, is inactive, promoting neither cell-killing nor cytokine release. There is not a gel shift with Y8A. Yet crystals that diffract x-rays do grow from a mixture of HLA-A2/Y8A and TCR proteins.

Preliminary x-ray data from these crystals show them to be isomorphous with the crystals containing wild-type peptide. If they are not isomorphous enough to simply use the phases from the solved structure, we should be able to readily solve, in the third year of this grant, the two new structures by molecular replacement techniques.

Year 3: September 1, 1996-July 31, 1997

We continued working on the crystals of the TCR/MHC complexes containing the mutant peptides LLFGaPVYV (Y5A) and LLFGYPVaV (Y8A) with the Y to A

changes shown in lower case. At this point (September, 1996), Yuan-Hua Ding, a postdoctoral fellow in the lab, joined the project. As both myself (David Garboczi) and Partho Ghosh planned to leave the Wiley lab in August 1997, Yuan-Hua Ding would be able to carry on the project.

Together Ding and I worked on obtaining better crystals of the the tax mutant peptide complexes. We prepared large crystals and collected x-ray data at CHESS. We also prepared crystals of a second and different TCR. This new TCR, called B7, also bound to the complex of HLA-A2 and tax peptide, but is a different amino acid sequence, both in the CDR loops and in the Valpha domain. The Vbeta domain is largely the same as the previously solved structure (6).

Experimental Methods

Both for the mutant peptide crystals and those of the B7 TCR, the same or similar techniques to those described for the previous years' work (see above) were used. These are, briefly, the overexpression in bacteria of the proteins in inclusion bodies, the refolding to form native protein, crystallization, and x-ray crystallography. The function of the proteins was assessed with gel-shifts on native polyacryamide gels.

The new crystals of the mutant peptide complexes were obtained by micro-seeding with newly-obtained seed crystals of the TCR/HLA-A2/wild-type tax peptide complex. They were about 400 um x 200 um x 150 um and large enough to collect x-ray data. Again crystals were grown of the TCR/HLA-A2 complex that contained the mutant peptide Y8A, even though this peptide will not lead to binding as seen in a gel-shift experiment. Crystals were frozen by immersion in liquid nitrogen and carried to the synchrotron frozen. Unfrozen crystals were also carried in the trays in which they grew.

Yuan-Hua Ding was able to work out methods of refolding the B7 TCR that yielded complexes of B7 and HLA-A2/tax peptide. This complex readily crystallized and was taken to the synchrotron also.

Results and Discussion

After producing a large number of crystals of the TCR/MHC complexes containing the mutant tax peptides, we traveled to the Cornell synchrotron (CHESS) to try to obtain x-ray diffraction data on the crystals. Datasets were collected on each of the mutant peptide-containing crystals. By inspection of the x-ray images as they were collected, we estimated that the resolution goes to about 3.0-3.5 Å. We were plagued by the high mosaicity of the crystals. These crystals appeared to be more consistently mosaic than the wildtype peptide-containing complexes. The datasets were stored on magnetic tapes and brought back from the synchrotron. As I (David Garboczi) and Partho Ghosh were leaving the Wiley laboratory to take up tenure-track jobs (at the National Institute of Allergy and Infectious Diseases, Rockville, MD and the UC San Diego, respectively), we did not get any farther in the analysis of the data from the mutant peptide-containing crystals.

Data was collected for the B7 TCR to a resolution of about 2.7 Å. Yuan-Hua Ding is working on the analysis of that data at present. The molecular replacement experiments are proving again to be quite dificult with this new TCR/HLA-A2 complex.

<u>Peptide Library</u>

During the last year of the grant, I also worked with a peptide library. With the structure of the TCR/MHC solved (6) and interpreted to the extent possible, I turned to thinking about how to obtain more information about the binding between a TCR and the MHC molecule. We saw that the specificity of a TCR for a

particular peptide was dificult to account for by looking at the structure. There just are not a lot of contacts and contact surface area between the TCR and the peptide.

We needed a way of addressing the question of what it is that is important in the TCR/peptide interaction. We saw that there was quite a bit of interaction between the TCR and the MHC molecule, but much less between the TCR and the peptide. One way to address this issue would be to find another peptide that would bind to HLA-A2 and to which this TCR would bind. Since the structure led us to state that the interaction between the TCR and MHC molecule does not look very specific, finding such a second peptide, especially if it was unrelated in sequence to the tax peptide, might be informative about what is important for the binding and thus the recognition of antigen by the T-cell.

I began a collaboration with Greg Weiss in the laboratory of Stuart Schreiber in the Department of Chemistry at Harvard University. We decided to make a soluble peptide library. We would randomize five positions of the tax peptide. The wildtype sequence is LLFGYPVFV. We made LLFxxxxxV, where "x" is all of the other amino acid residues except for cysteine and obtained a yield of about 100 mg of peptide. This replacement of five positions with 19 amino acids implied that the diversity of the library was about 2.5 million. We were able to refold HLA-A2 with this peptide library and the resulting HLA-A2 crystallized into long needles that look almost good enough for x-ray diffraction. The idea would be to mix the TCR protein with the library-bearing HLA-A2, to try to isolate the complexes, and eventually to identify the peptides. Then an x-ray structure of a TCR in complex with HLA-A2 and this new peptide could be done. These experiments have also been interrupted by my leaving the Wiley lab to begin a laboratory at the NIH.

Recommendations in relation to the Statement of Work

The Statement of Work states that we believed we would solve the TCR/MHC complex by the method of molecular replacement. As described above, that was not possible even though we had protein models already for HLA-A2 (9), a mouse beta subunit (10), and a mouse Valpha domain (11). This was primarily because of the data quality of these crystals, but we were able to go forward with the technique of multiple isomorphous replacement and finish the structure (6). The high mosaicity of these crystals prevent high quality x-ray data from being obtained. Further work could be done to obtain crystals that are not as mosaic. Making modifications of the TCR protein would be the first experiment I would recommend. Since the constant domains of the TCR are not well-resolved in the complex, their removal may be a way of modifying the TCR in pursuit of better crystals.

The Statement of Work states that the binding affinity between the TCR and HLA-A2 will be measured. We were not able to measure binding due to the lability of the TCR protein. The interaction between the alpha and beta subunits of the TCR as produced by us is not covalent, since the disulfide bond between the subunits was removed to allow the overproduction of sufficient and pure protein for crystallography experiments. Upon dilution of the TCR, these non-covalently bonded alpha and beta chains dissociate. It appears from our observations (unpublished) that upon dissociation, the alpha chain aggregates with itself and precipitates. Away from the complex, the alpha chain is also susceptible to proteolytic cleavage and we have seen degradation products of alpha on SDS gels loaded with the complex. It seems that a covalent link of some kind is needed between the subunits in order to measure binding between the TCR and HLA-A2.

Conclusions

In this 3-year postdoctoral fellowship grant, we have been able to solve the structure of a T-cell receptor in complex with a MHC protein, HLA-A2, and a viral peptide (6).

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Table I. Structural analysis

Data Sets	Resolution limit (Å)	Mosaicity	R_{merge}‡	Comp† (red)	I/σ_I	R_{deriv}‡ (Sites)	R_{cullis}‡	f_h/ε§	fom†† (res)
A. C2 (Z=1)*									0.41 (3.5 Å)
Native	2.90	2.5°	13.9	82 (3.7)	7.3				
L1C + EMP	3.10	1.0°	10.1	98 (2.5)	8.2	22.4 (1)	75	1.13	
K91C + EMP	3.20	2.4°	8.6	81 (2.7)	9.2	21.6 (1)	89	0.79	
Y67C/K91C + EMP	2.60	1.1°	10.9	95 (3.1)	7.0	27.2 (2)	85	0.94	
B. C2 (Z=1)*									0.35 (3.5 Å)
Native	2.80	1.5°	11.8	79 (1.8)	6.4				
L1C + EMP	3.40	0.6°	10.7	75 (1.8)	6.3	23.5 (2)	76	0.94	
K_2PtCl_4	3.60	1.4°	11.8	93 (2.5)	7.0	34.5 (5)	82	0.92	
C. $P2_1$ (Z=2)*									0.30 (3.7 Å)
L1C/11C Native	3.75	3.0°	14.3	86 (2.6)	6.6				
L1C/11C + EMP	3.40	2.5°	13.9	85 (2.6)	6.5	35.4 (2)	80	1.12	

Molecular Replacement§§	HLA-A2/Tax9	TCR Vα	TCR Vβ	TCR Cβ
Rotation (10-3.8 Å)	7.3σ	2.3σ	4.3 σ	3.4σ
Translation (15-3.8 Å)	10.3σ	8.5σ	10.3σ	5.7σ

Refinement Resolution	Reflections (free)	Atoms	R_{free}‡	R_{cryst}‡	Bonds‡‡	Angles‡‡	B-factors¶
6.0-2.6 Å	26273 (3006)	5716	32.2	24.0	.013 Å	1.81°	4.8 Å^2

Domains	$\alpha_1\alpha_2$	α_3	β_2m	Tax9	Vα	Vβ	Cβ
Mean B-factors	27 ± 15 Å^2	81 ± 32 Å^2	29 ± 16 Å^2	19 ± 7 Å^2	36 ± 15 Å^2	36 ± 15 Å^2	65 ± 18 Å^2
Real-Space Fit**	0.87 ± .03	0.74 ± .15	0.86 ± .09	0.89 ± .02	0.87 ± .05	0.88 ± .03	0.80 ± .12

*Space groups of crystal forms and derivatives are described in Methods. Z, number of MHC/Tax/TCR complexes per asymmetric unit.

‡$R_{merge} = 100 \times \frac{\sum_h \sum_i |I_{h,i} - I_h|}{\sum_h \sum_i I_{h,i}}$, where I_h is the mean intensity of symmetry-related reflections, $I_{h,i}$.

$R_{der} = 100 \times \frac{\sum_{hkl} |F_p - F_{ph}|}{\sum_{hkl} F_p}$, where F_p is the native amplitude and F_{ph} is the derivative amplitude. Sites, number of heavy atom sites per asymmetric unit.

$R_{Cullis} = 100 \times \frac{\sum_{hkl} ||F_p \pm F_{ph}| - F_{h\,calc}|}{\sum_{hkl} |F_p \pm F_{ph}|}$, where F_p is the native amplitude, F_{ph} is the derivative amplitude for centric reflections, and $F_{h\,calc}$ is the calculated heavy atom structure factor.

$R_{free} = 100 \times \frac{\sum_{hkl} |F_{obs} - F_{calc}|}{\sum_{hkl} F_{obs}}$, where R_{free} is calculated for a randomly chosen 10% of reflections ($F > 0$) omitted from refinement, and R_{cryst} is calculated for the remaining 90% of reflections ($F > 0$) included in refinement.

†Comp = 100 x (number of observed unique reflections)/(number of possible unique reflections). red = (number of observed reflections)/(number of observed unique reflections).

§ fH / e is the phasing power, with fH being the mean amplitude of the heavy atom structure factor and e the r.m.s lack-of-closure error.

††fom, figure of merit. res, resolution to which figure of merit is determined.

§§Rotation and translation solution peak heights above the mean in rotation and translation function maps.

‡‡r.m.s. deviations from ideal values.

¶r.m.s. deviation between B-factors of bonded atoms.

**Real-space fit correlation coefficient [45] calculated from 2Fobs-Fcalc electron density and the refined model of MHC/Tax/TCR.

TABLE 1 (continued)

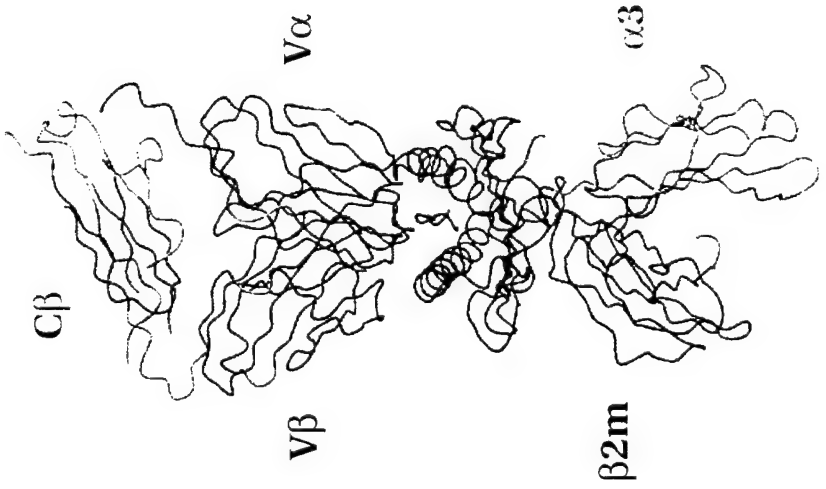

Fig. 1. Intercellular complex of alpha/beta TCR (top) and the HTLV-1 Tax peptide bound to HLA-A2 (bottom) shown as an alpha-carbon trace, colored by temperature factor: blue is low and red is high temperature factor (greater than 60 square angstroms) and shows less ordered regions. The TCR constant region of alpha is not shown.

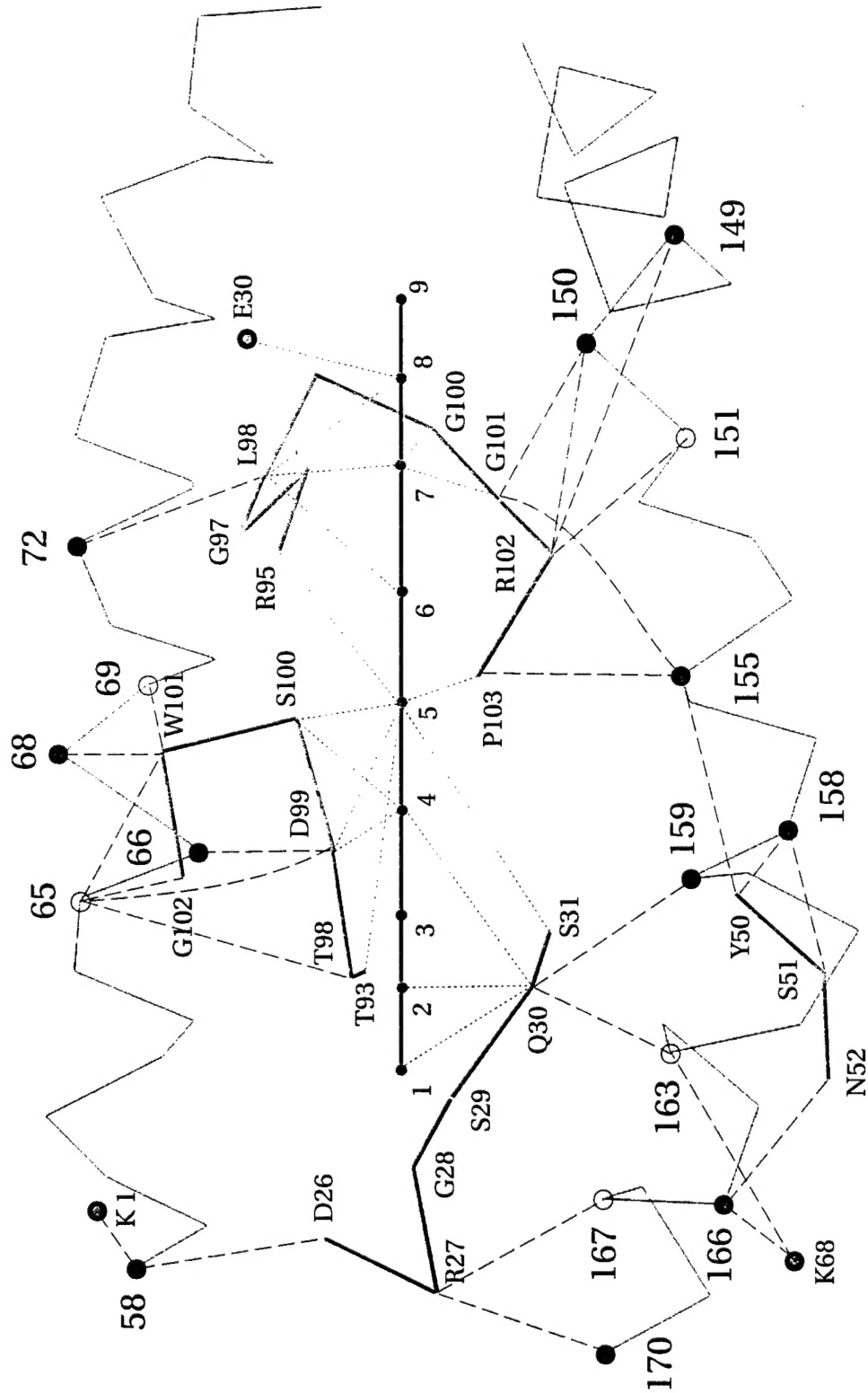

Fig. 2. Interaction of TCR with the Tax peptide and HLA-A2. CDR residues (solid red lines) contact HLA-A2 (green dashed lines) and Tax peptide (red dotted lines). Human MHC class I conserved (solid circles) and polymorphic (open circles) residues are shown. For clarity, the peptide is depicted as a straight line and contact between Valpha30Q and HLA-A2 is not shown.

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List of Personnel Receiving Pay from this effort

David N. Garboczi, Ph.D.